Circadian Clocks

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Circadian clocks: **Omnes viae Romam ducunt**

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The circadian clock in all organisms is so intimately linked to light reception that it appears as if evolution has simply wired a timer into the mechanism that processes photic information. Several recent studies have provided new insights into the role of light input pathways in the circadian system of *Arabidopsis*.

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To cope with the regular daily changes in their environment, organisms, from cyanobacteria to humans, have evolved an endogenous clock that anticipates the changes and programs their physiology accordingly. One of the most conspicuous circadian features is the ability to maintain approximately one-day oscillations in constant conditions, known as a free-running clock. Depending on the organism and the nature of the constant artificial environment it is exposed to — the light intensity or colour, temperature, nutrient composition and so on — the free-running period (τ) can range from approximately 19 to 29 hours. Although these periodicities are extremely precise from cycle to cycle, they do not accurately represent the 24 hour day. In nature, therefore, circadian clocks have to be synchronised on a daily basis. The environmental signals used for this 'entrainment' are called *zeitgebers*, and light appears to be the most dominant *zeitgeber*.

Experiments that were designed to identify the circadian photoreceptor — for example, analyses of action spectra or of mutants with altered circadian behaviour — show that the clock can recruit light information from several redundant receptors and pathways [1–3]. To complicate matters, light entrainment pathways turn out to be themselves under clock control [1,4,5], and genes that are essential for clock function turn out to be closely linked to light transduction mechanisms [6]. Five recent papers [7–11] investigating the circadian system in the higher plant *Arabidopsis* take this complexity even further. The *Arabidopsis* clock remains entrainable by light even when four known photoreceptor genes — *phyA*, *phyB*, *cry1* and *cry2* — are 'knocked out' in a quadruple mutant [7], and likely candidates for additional light inputs to the clock have been identified [8,9]. An *Arabidopsis* mutant (*toc1-1*) shows reduced light responses — in this case to the photoperiod — even though the gene and its product are not even light responsive [10]. Finally, unlike in animals, the different cellular clocks in different parts of the plant body appear to function with complete autonomy [11].

Shining light on the clock

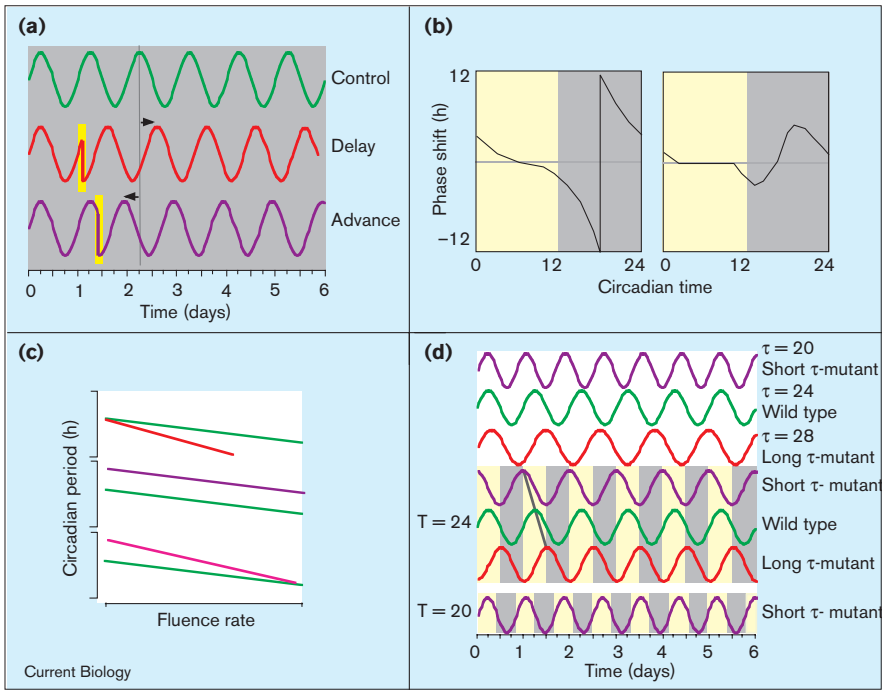
Circadian clocks can be reset by a single light pulse. This means that some variable of the mechanism that generates the endogenous rhythmicity must directly or indirectly be affected by light — for example, by a decrease in its concentration. Depending on the phase of the oscillation — the 'circadian time' — a light pulse will either delay or advance the oscillator (Figure 1a). These phase-specific light responses are the bases for entrainment and can be represented by what is known as a phase response curve (by definition delays are negative and advances positive). In the example shown in Figure 1a, the concentration of the representative clock variable is always reduced by a light pulse to its minimum. This would lead to a strong phase response curve, as shown in Figure 1b on the left. In most higher organisms, however, the maximum phase shifts are much smaller (Figure 1b, right).

The shape of its light phase response curve determines how a clock is entrained — for example, its relative phase in a light:dark cycle. Furthermore, the light phase response curve determines a system's τ in different fluence rates of constant light; this can be represented in what are called fluence-rate response curves (Figure 1c). Depending on the amplitude of the phase response curve, and on the ratio between advances and delays, τ will either shorten (when advances greatly exceed delays) or lengthen (when delays are equal to or greater than advances) with increasing light fluence rates. The two oscillators shown in the top graph of Figure 1c have the same period in constant darkness; one of them, however, is more accelerated by light than the other — for example, its phase response curve has a larger advance region. In the middle graph, the two oscillators have different periods in constant darkness, but the same light phase response curves — hence the parallel slopes. And in the bottom graph, they have both different periods in constant darkness and different light phase response curves.

A clock for all seasons

Clock mutants carrying different alleles of the affected gene often differ in their free-running periods (Figure 1d). Even if the light phase response curves of these 'τ mutants' are identical, they will entrain differently to a 24 hour light:dark cycle — mutants with short periods will lead those with longer periods (Figure 1d). Different physiological states will, therefore, be exposed to light and darkness. This is not only true for τ mutants

Figure 1



How light affects the circadian clock. **(a)** Single light pulses (yellow bars) directly or indirectly change – in this example, decrease – the concentration of a component in the rhythm generator (see also Figure 2). Depending on the phase of the endogenous rhythm, light can delay or advance the progression of the clock (arrows). For reference, a vertical line is drawn through a control that did not receive a light pulse. **(b)** Phase-response curves are constructed from the respective delays (negative) and advances (positive) elicited by light pulses at different times during the cycle (the 'circadian time'). The examples shown in (a) would give rise to large phase shifts, as shown in the phase response curve on the left, but weaker phase shifts are common in most higher organisms, as illustrated by the phase response curve on the right. **(c)** The shape of the phase response curve determines the differences in circadian period (τ) caused by constant light at different fluence rates (see text). **(d)** The circadian clock entrains with different phase relationships to a 24 hour light:dark cycle (T = 24), depending on τ in constant conditions. The top three time series show three free-running rhythms for an arbitrary wild type, assumed to have a 24 hour period, and short and long τ mutants. The effect of variations in τ on entrainment is illustrated in the middle three traces, where for reference a line is drawn through the peaks of the rhythms. But when a short τ mutant is entrained by a short light:dark cycle – for example, 10:10 hours, with the total period of one cycle T = 20 – its phase relationship is similar to the wild type with a T = 24 cycle.

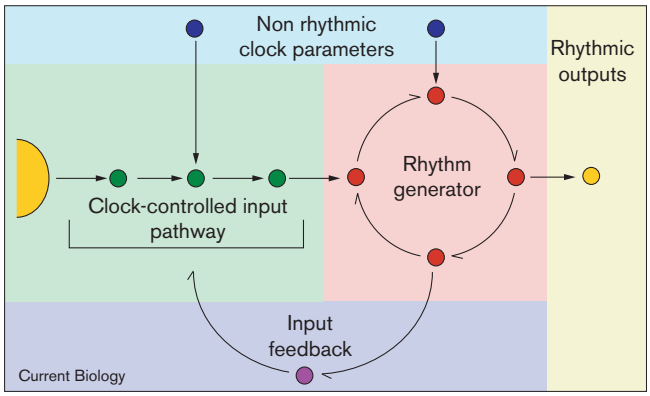
but also for organisms in nature as day-length varies over the course of the year, if they live far enough from the equator. This time-of-year-specific light exposure provides a basis for the photoperiodic response which, for example, determines flowering.

Wild-type *Arabidopsis* plants take longer to flower in short days than in long days, whereas the *toc1-1* mutant is far less choosy about day length [12]. This reduction in photoperiod response is not due to altered light responses, which appear to be unchanged in this mutant. Although wild-type and *toc1-1* mutant plants show different periods in constant darkness — 22.3 hours for the *toc1-1* mutant versus 27.5 hours for wild type [10] — their fluence-rate response curves show parallel slopes, similar to the middle graph in Figure 1c [12]. Because of its shorter period, the *toc1-1* mutant entrains with an earlier phase than wild type in light:dark cycles (illustrated by the T=24 lines in Figure 1d, where T is the total length in hours of a light:dark cycle). If this altered light exposure is responsible for the change in photoperiod response, it should be rescued when exposed to a shorter light:dark cycle (illustrated by the T=20 line in Figure 1d), because under these conditions a short period mutant would entrain with a phase angle similar to wild type. A strong photoperiodic response is rescued in the *toc1-1* mutant when the plants are grown in short cycles (T=21). Like wild-type plants, the mutant plants take longer to flower in short days, with a light:dark cycle of 7:14 hours, than in long days, with a light:dark cycle of 14:7 hours.

Placing gene products within the clock

Because mutation of the *TOC1* gene has no apparent effects on light responsiveness, its product is unlikely to be part of a light input pathway. The short period must have another cause. The *TOC1* gene is expressed rhythmically and it encodes a nuclear protein, thought likely to be involved in transcriptional regulation, which has sequence motifs similar to those found in the 'two-component' signal transduction systems much used by prokaryotes [10]. Because mutation of the gene affects the rhythmicity of its own expression, *TOC1* is either part of the rhythm generator itself (Figure 2), or part of a signal transduction pathway — obviously not for light — that is both an input to and output from the rhythm generator. Other *Arabidopsis* mutations of genes which clearly are part of a light input pathway — *phyA*, *phyB*, *cry1* and *cry2* — also have circadian effects [3,7]. The *phyA* mutants show altered fluence-rate response curves, similar to those shown at the bottom of Figure 1c [3]. The same is true for the *cry1* mutant in blue light, while *phyB* in red light resembles more the example illustrated at the top of Figure 1c, and *cry2* appears to have no effects on period.

Figure 2



The domains of the circadian system. At the heart of a circadian system, a mechanism generates the rhythmicity, possibly via some negative feedback loop (red area). Note that this generator on its own can theoretically produce a rhythm outside of the circadian range. The circadian period can be tuned by other components of the system. For entrainment with the 24 hour day, input pathways transduce environmental information – the *zeitgeber*, 'time giver' – that resets elements of the rhythm generator (green area). Input pathways can be themselves under the control of the rhythm generator. Clock controlled input pathways (purple area) – the *zeitnehmer*, 'time taker' [1] – influence the period length and robustness of the oscillating system. All components of the rhythm generator and the *zeitnehmer* loop are rhythmic, in addition to those of a clock-controlled input pathway. The outputs of the system are obviously also rhythmic (yellow area). Although they are shown here as originating from the rhythm generator, the outputs could theoretically be controlled by any rhythmic element of the system. In addition to the rhythmic components, other non-rhythmic elements can be essential for circadian function. Of the genes discussed in the text, *PHYB* and perhaps also *FKF1* are likely to be part of a clock-controlled input; *ZTL* is likely to be a non-rhythmic parameter; while *TOC1* could either be part of a rhythmic input loop or the rhythm generator.

Although these genes are clearly involved in phototransduction, they could still affect τ in constant darkness (τ_{DD}). Light input pathways — as well as non-photic input pathways of the clock — are often under circadian control, closing a loop between input elements and the rhythm generator [1,13]; they could therefore influence the progression of the clock both in constant light and constant darkness (Figure 2). Whether the four *Arabidopsis* light input pathway components mentioned above have an influence on τ_{DD} is still not known, because long-term measurements by a luciferase reporter in constant darkness have only recently become possible [10]. By extrapolation from the fluence-rate response curves for *phyA-201* mutant and wild type plants [3], however, different values for τ_{DD} are possible.

The recent construction of *Arabidopsis* double and quadruple mutants for *phytochromes* and *cryptochromes* [7] clearly shows, firstly, that these light input pathway components are not essential for generating the circadian rhythmicity, as the mutant is rhythmic, and secondly, that additional light inputs must exist, as the mutant is entrainable in light:dark cycles. The quadruple mutant has an apparently normal τ at higher constant light fluence rates, but as no fluence-rate response curves were measured, it remains possible that τ is affected at low fluence rates, as reported for the *phyA* single mutant, and even in constant darkness. Additional candidates for providing circadian light inputs could be among the remaining three phytochromes in *Arabidopsis*, as well as the products of the recently identified genes *ZEITLUPE* (*ZTL*) [8] and *FKF1* [9]. *ZTL* is constitutively expressed and its mutant shows a fluence-rate response curve similar to the one at the bottom of Figure 1c, as well as delayed flowering. In contrast, *FKF1* expression is rhythmic and its mutant has a τ that is not significantly different from wild type, at least not in constant light, though it does also show delayed flowering.

The number of plant genes known to influence the circadian clock, either as part of an input pathway or as a potential component of the rhythm generator, seems to increase almost exponentially. While molecular research in animals claims to have practically closed the circadian loop [14,15], the loop in plants is only starting to open up, and we already get a glimpse of the enormous molecular complexity of the circadian network. Many roads lead to Rome and many light input pathways feed light information to the clock. Rome was a critical centre for the mediaeval catholic empire, but without the many (Rome-controlled) roads connecting it to the world, the Empire could not have functioned properly. By analogy, there is surely some central mechanism that generates the endogenous rhythmicity, but without the clock-controlled input pathways, the clock also cannot function normally [13].

Placing clocks within the organism

The circadian clock in mammals resides in the suprachiasmatic nucleus (SCN). In spite of the fact that molecular circadian clocks have been identified in many peripheral tissues and cells, the role of the SCN as a central pacemaker is unchallenged [16]. Although the different body clocks take different times to adjust to a new time zone — hence the negative effects of jetlag — light entrainment occurs only through the eyes and the SCN [2]. This hierarchical structure does not exist in plants, because different parts of a plant can be entrained independently by different light:dark cycles — for example, the tip of the leaf could be entrained to New York time, while the rest of the plant is entrained to London time. These unnatural phase relationships are even maintained when the plant is released to constant light [11]. It thus seems that no communicating agents couple the different cellular clocks within a plant — at least not when the output reporters are driven by the promoters of the rhythmically expressed genes for chlorophyll a/b-binding protein, phytochrome B1 or chalcone synthase, as in the experiments of Thain *et al.* [11].

This is surprising in view of the fact that plants, like animals, have to coordinate different anatomical parts in many ways — for example, in the regulation of turgor — and it would seem unlikely that temporal regulation would be exempt from this requirement for coordination. The resolution to this enigma could be either that plants can rely on similar (but independent) entrainment of the entire organism, or alternatively that the circadian program of plants consists of several clock systems — one ticking autonomously in every cell and another responsible for temporal programs that have to coordinate different anatomical parts over the course of the day. The latter is not unlikely because independent circadian oscillators have already been shown to exist even in a unicellular alga [17].

Acknowledgements

We thank Millar and Somers for helpful comments on the manuscript. The title of this dispatch is the Latin version of the proverb "all roads lead to Rome"; this was originally "mille viae ducunt homines per saecula Romam, qui dominum toto querere corde volunt", which translates as "a thousand roads lead humans for centuries to Rome seeking God with all their heart", from a collection of mediaeval proverbs (*Liber parabolorum*, by Alanus ab insulis, 1125–1205). This article is dedicated to Peter Schwartze.

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